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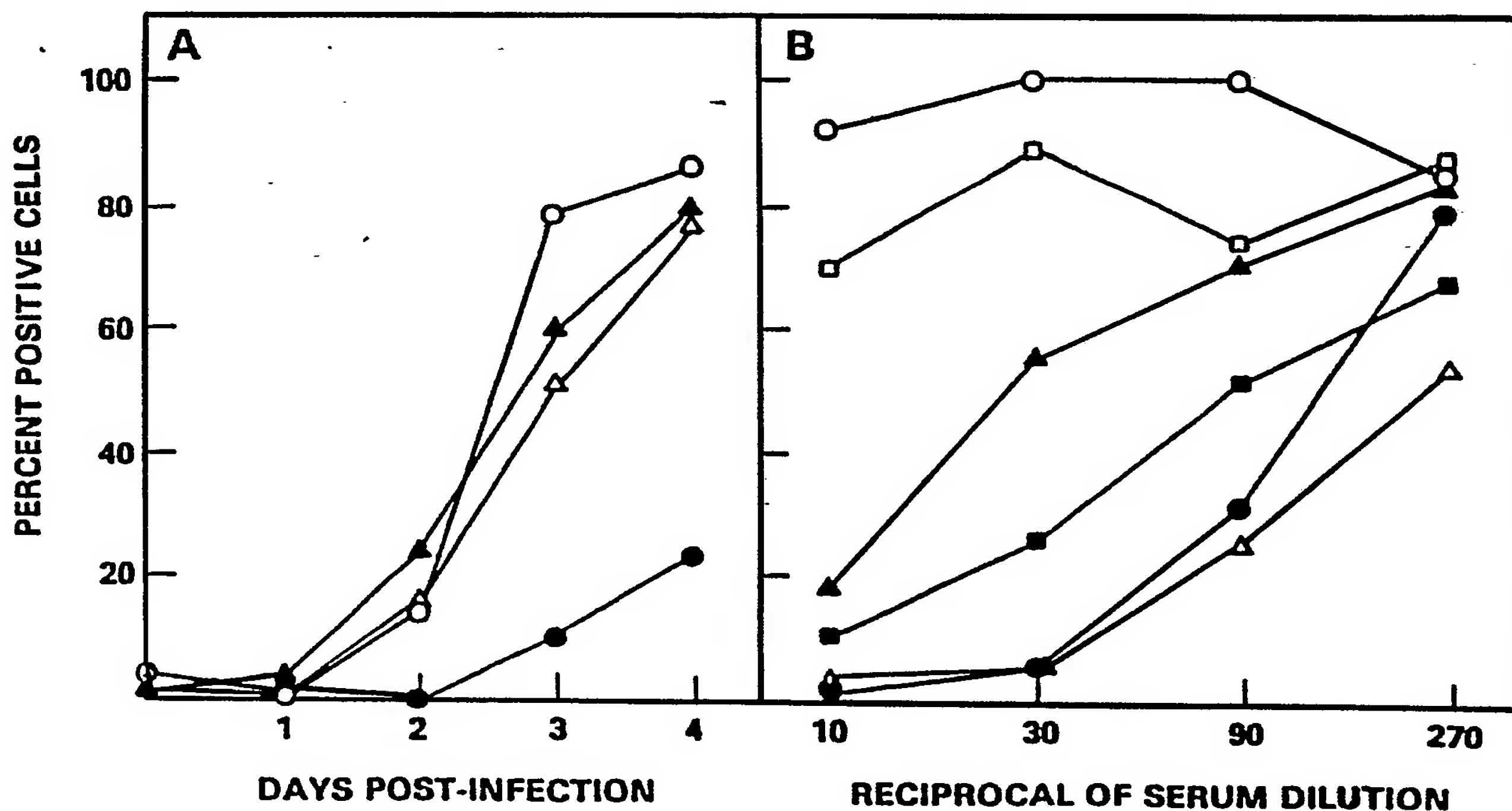
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(54) Title: A METHOD FOR DETECTING HTLV-III NEUTRALIZING ANTIBODIES IN SERA



(57) Abstract

Method of measuring natural human antibodies in sera which will neutralize HTLV-III infection in an *in vitro* assay. Cell-free virus is incubated with serum and used to infect H9 cells, which are then put in a culture for three days. The viral infectivity is then assayed using a monoclonal antibody specific for HTLV-III p24 in an immune fluorescent assay.

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A METHOD FOR DETECTING HTLV-III NEUTRALIZING ANTIBODIES IN SERA

Background

During the recent past in 1984 the virus HTLV-III emerged as the most probable causative agent of acquired immune deficiency syndrome (AIDS) illness (see Gallo, et al, in the Material Information Disclosure, post 1, 2, 3). Also when an H9 cell is suitably infected with the HTLV-III virus and cultivated, an immortalized product results. The HTLV p24 core antigen has been isolated and purified from the immortalized H9/HTLV-III cell line (Gallo, et al, Serial No. 635,610 filed July 30, 1984, "Isolation of p24 Core Protein of HTLV-III").

Generalized Process

In the present invention the natural antibodies in sera are assessed for their ability to neutralize HTLV-III infection. HTLV-III infection is monitored by following expression of the viral core protein, p24, by means of a specific monoclonal antibody to HTLV-III p24.

Also in the present invention the effect is to use sera with accompanying antibodies within it to effectively neutralize an amount of virus. This antibody neutralization may be either in whole or in part and a quantitative estimate of neutralizing antibody titer may be made using the outlined procedures. The method is applicable to serum from any species and hence is useful for assessing potential vaccine preparations for effectiveness in eliciting an HTLV-III neutralizing antibody response. In the last step, when it is not possible to observe viral infection within three days, then the serum has neutralized the viral infectivity in toto. It is believed that the neutralizing antibody in the sera bind to the viral envelope glycoprotein which is responsible for the initial attachment of the virus to the receptors and, thus, blocks the infective action of the virus.

Also, the effort is made to utilize for a

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special purpose assays for HTLV-III dependent on antigen-antibody reaction and the presence of antibodies in sera of AIDS and related patients which neutralize viral antigen and are useful for protection.

5 Sketch I shows the process of the present invention.

Sketch I

Virus (HTLV-III)

+

10 Serum (with or without neutralizing antibodies)



Binding of specific antibody to viral antigen

Infect H9 cells and cultivate (3 day hold)



Assay for virus infectivity by monitoring expression of HTLV-III p24

15 Material Information Disclosure

- 1) Sarngadharan, et al, "Antibodies Reactive with Human T-lymphotropic Retroviruses (HTLV-III) in the Serum of Patients with AIDS," Science, 224:506-508, 1984.
- 20 2) Safai, et al, "Seroepidemiological Studies of Human T-lymphotropic Retrovirus Type III in Acquired Immunodeficiency Syndrome," Lancet, i, 1438-1440, 1984.
- 3) Gazzolo, et al, "Antibodies to HTLV-III in Haitian Immigrants in French Guiana," New Engl. J. Med., 311:1252-1253, 1984.
- 25 4) Clumeck, et al, "Seroepidemiological Studies of HTLV-III Antibody Prevalence Among Selected Groups Heterosexual Africans," to be presented at the International Conference on AIDS, Atlanta, April 14-17, 1985.
- 30

- 3 -

- 5) Gonda, et al, "Sequence Homology and Morphologic Similarity of the AIDS Virus, Human T-cell Lymphotropic Virus Type III (HTLV-III), and Visna Virus, Member of the Pathogenic Lentivirus Subfamily," Science, in press.
- 6) Robert-Guroff, et al, "Detection of the Human T-cell Lymphoma Virus p19 in Cells of Some Patients With Cutaneous T-cell Lymphoma and Leukemia Using a Monoclonal Antibody," J. Exp. Med., 154:1957-1964, 1981.
- 7) U.S. Serial No. 635,610, Gallo, et al, filed July 30, 1984, "Isolation of p24 Core Protein of HTLV-III."

None of the above references disclose the present method for detecting natural antibodies in sera which neutralize HTLV-III and protect therefor and measure the residual viral infectivity with a specific MAB such as anti-p24 HTLV-III (BT3 Biotech Research Labs, Veronese et al submitted).

The Invention

The isolation of the human T-cell leukemia (lymphotropic) virus type III (HTLV-III) from cells of numerous patients with the acquired immunodeficiency syndrom (AIDS) presented the first evidence that the virus was the etiologic agent of the disease. This conclusion has been strengthened by the results of many subsequent investigations including those of sero-epidemiologic studies which showed the presence of HTLV-III specific antibodies in the serum of the vast majority of patients with AIDS and AIDS-related complex (ARC). In addition, viral specific antibodies have been found in the serum of every group originally identified as a risk for AIDS, including homosexual males, hemophiliac recipients of factor VIII, intravenous drug users, and Haitians. More recent and wide-ranging serologic studies have identified additional populations exposed to the virus including heterosexual partners of AIDS or ARC

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patients and individuals from certain regions of Africa, especially Zaire and Rwanda, where AIDS as well as HTLV-III appear to be endemic.

5 While these sero-epidemiologic studies have provided many insights into the mode of transmission and extent of HTLV-III infection, there have been no reports concerning possible protective or therapeutic effects of HTLV-III specific antibodies in sero-positive individuals. Therefore an investigation was conducted to
10 determine if AIDS and ARC patients possess antibody activities capable of inhibiting viral infection. Such a natural defense mechanism enables an infected host to avoid cell to cell spread of the virus and, hence, progression of the disease may be retarded or prevented. In
15 several animal retroviral systems, neutralizing antibodies have been described which bind to the viral envelope glycoprotein which is responsible for initial attachment of the virus to the target cell receptor (Steeves, R.A., et al, J. Virol., 14:187-189, 1974). By
20 blocking the binding of virus to this receptor, virus neutralizing antibodies may effectively inhibit viral infection. In the studies reported here, it was asked whether HTLV-III elicited specific neutralizing antibodies in AIDS or ARC patients and whether any protective
25 effect of such antibodies could be demonstrated.

The H9 clone of the HT cell line (specific process and examples) was used as target for cell-free HTLV-III infection, and several sera were initially analyzed for virus neutralizing antibody activity.
30 Infection of the H9 cells was assessed by monitoring the expression of HTLV-III p24 using a monoclonal antibody in an indirect immune fluorescence assay. Figure 1 illustrates the kinetics of infection of H9 cells with HTLV-III virus preincubated with sera positive or negative for virus neutralizing activity. By three days post-
35 infection, approximately 80% of the H9 cells incubated with HTLV-III pretreated with serum of a healthy normal

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donor were infected as indicated by their expression of HTLV-III p24. In contrast, only 10% of H9 cells expressed HTLV-III p24 at day three when infected with virus pretreated with serum from a patient with ARC. That this inhibition of infection was mediated by a viral rather than a cellular antigen was shown by ready infection of H9 cells with HTLV-III following pre-treatment of the cells rather than the virus with the same sera (Figure 1a). The inhibitory activity of certain sera was not simply a non-specific effect of high serum concentrations because the activity was titratable. As illustrated by the several sera titrated in Figure 1b, sera possessing inhibitory activity were found in all categories of patients and healthy members of groups at risk for AIDS.

In order to confirm that the inhibitory activity detected was directed against a viral rather than a cellular antigen, sera were absorbed with preparations of cell-free virus or with infected or uninfected H9 cells. While absorption with cells had little effect, absorption with viral preparations substantially decreased titers of sera with inhibitory activity as shown in Table 1 below.

Natural antibodies capable of neutralizing HTLV-III infection of H9 cells were detected in 60% of adult AIDS patients and in 80% of adults with ARC, but in 0% of normal healthy heterosexual controls. Geometric mean antibody titers were two-fold higher in ARC patients compared to AIDS patients and were even higher in 2 antibody positive healthy homosexuals. This finding suggests that virus neutralizing antibodies may exert some in vivo protective effect. The presence of these antibodies indicates an immunologic response to HTLV-III which may be utilized for therapeutic advantage. Also, the methodology employed in these studies can be directly useful in monitoring future vaccine approaches.

Therefore, having defined a system in which serum IgG could neutralize the infectivity of HTLV-III

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for H9 cells by binding to the virus, a number of human sera were analyzed for this antibody activity. The results are summarized in Table 2 below. It is clear that a high prevalence of patients with either AIDS or ARC possess virus neutralizing antibodies in contrast to healthy heterosexual individuals in which no such activity was demonstrated. While antibody titers ranged upwards of 500 for both patient groups, overall titers were low. However, it was observed that ARC patients possessed a two-fold higher geometric mean antibody titer compared to that of the AIDS patients studied. It was also seen that among healthy homosexuals for risk for development of AIDS, the geometric mean antibody titer, albeit determined on only 2 antibody positive individuals, was substantially higher than that of either of the two patient groups. This trend of higher titer with less or insignificant disease manifestations suggests a protective effect of the neutralizing antibodies.

HTLV-III neutralizing antibody activity was not detected in any normal healthy heterosexual individuals (Table 2). However, a barely detectable titer (of 13) was obtained in one of 4 serum samples from patients with acute mononucleosis. This result may suggest some weak cross-reactivity with viral antigen in sera possessing high levels of heterophilic antibodies.

In other retroviral systems, the major envelope glycoprotein is the target for neutralizing antibody. These naturally occurring virus neutralizing antibodies may be meaningful with regard to an in vivo protective effect.

The results of the present invention show a trend that individuals with less severe disease or those infected with HTLV-III but not yet manifesting clinical symptoms, possess higher neutralizing antibody titers. This suggests a human vaccine approach may be worthwhile. On the other hand, it is interesting to speculate that the role of neutralizing antibody in the overall biology

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of HTLV-III may be similar to that found in the visna virus system. Infection with visna virus, a non-oncogenic retrovirus which causes a slowly progressive disease in sheep affecting primarily the lungs and central nervous system, is persistent. It has been shown that neutralizing antibodies elicited by the virus have a narrow range of specificity which cannot inhibit infection by mutant viruses which arise during the course of the disease. Thus, the neutralizing antibodies exert a selective pressure, leading to replication of non-neutralized mutant viruses. It is also of interest that "early sera" taken from relatively recently infected animals possess a more restricted neutralization range compared to "late sera" obtained from animals infected for more than three years. These "late sera" were able to neutralize a broader range of visna mutants including all ancestral strains. This is relevant to HTLV-III especially because of the demonstrated genomic variability from isolate to isolate, particularly in the viral envelope region and also because of the demonstrated relatedness of HTLV-III to visna virus (Gonda, et al, Science, in press).

The demonstration of HTLV-III neutralizing antibodies in sera of patients with AIDS and ARC and in healthy individuals infected with HTLV-III is a meaningful finding which demonstrates an immunologic response during the course of disease development which may be utilized for therapeutic advantage. It furthermore indicates that appropriate vaccine approaches may be effective in preventing viral infection from the outset. The methodology described here will be useful in monitoring these future procedures and will also be useful in additional basic investigations concerning the biology of HTLV-III infection. Further studies will determine whether the presence of virus neutralizing antibodies in patient sera have any prognostic value or will be indicative of appropriate treatment regimens.

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Description of the Figures

Sera from a normal healthy heterosexual (o) and from a patient with ARC (●) are compared in Figure 1A. In a parallel experiment, these same sera were preincubated with H9 cells for 1 hr. at 4°C. The cells were washed with PBS, incubated with the cell-free HTLV-III preparation, and cultured as in Example 2. Results of this treatment of the H9 cells with sera are represented for the normal, healthy heterosexual serum (Δ) and the serum of the patient with ARC (▲).

In Figure 1B representative titrations are shown for two sera negative for viral neutralizing antibody activity: a patient with AIDS (o) and a patient with ARC (□). Representative sera positive for virus neutralizing antibody were obtained from a pediatric AIDS case (●), a patient with ARC (■), a healthy homosexual (Δ), and an adult AIDS patient (▲). All values obtained were normalized to the level of infection attained in the presence of a standard antibody-negative serum.

TABLE 1

HTLV-III Neutralizing Activity is a Property of IgG and is Directed Against a Viral Antigen

<u>Serum Samples</u>	<u>Patient Diagnosis</u>	<u>Serum Treatment (a)</u>	<u>HTLV-III Neutralizing Antibody Titer (b)</u>
Experiments with Purified IgG:			
1	ARC	None Purification of IgG	115 110
2	AIDS	None Purification of IgG	34 40
3	ARC	None Purification of IgG	60 76
Absorption Experiments:			
4	ARC	None Absorbed with HTLV-III Absorbed with H9 Cells Absorbed with H9/HTLV-III	135 < 10 120 90
5	AIDS	None Absorbed with HTLV-III Absorbed with H9 Cells Absorbed with H9/HTLV-III	75 < 10 22 34

TABLE 1 (continued)

Serum Samples	Patient Diagnosis	Serum Treatment (a)	HTLV-III Neutralizing	
			Antibody Titer (b)	
6	ARC	None		> 270
		Absorbed with HTLV-III		50
		Absorbed with H9 Cells		> 270
		Absorbed with H9/HTLV-III		> 270

AIDS = acquired immunodeficiency syndrome

ARC = AIDS related complex

a) IgG was purified from 0.5 ml aliquots of human serum by absorption to protein A-Sepharose equilibrated in PBS. Following extensive washing of the columns with PBS, IgG was eluted with 0.1 M glycine-HCl, pH 2.8. the eluate was neutralized with 2M Tris-HCl, pH 8.0, dialyzed extensively against 10 mM ammonium bicarbonate, and lyophilized. The purified fractions were dissolved in 0.5 ml PBS, filter sterilized, and diluted in media for titration of neutralizing antibody activity as described in Example 2.

For virus absorption experiments, 62 ml of cell-free virus supernatant containing 2 to 5 x 10⁸ virus particles/ml were pelleted as described in Example 2. The viral pellet was resuspended in 100 l of a 1:10 dilution of serum to be absorbed and incubated overnight at 4°C. The virus was again pelleted by centrifugation and the absorbed serum was saved for titration of virus neutralizing antibody activity. Sera were similarly absorbed on pellets of washed 10⁷ cells and titered.

b) Values for percent of HTLV-III p24-positive cells were normalized to the level of infection obtained in the presence of a standard negative serum treated similarly as the test serum. Antibody titers were then expressed as the reciprocal of the serum dilution at which virus infection was 60% of that obtained in the presence of this standard negative serum.

TABLE 2

HTLV-III Neutralizing Antibody in AIDS and ARC Patients and Others at Risk^a

<u>Serum Source</u>	<u>No. Positive/ No. Tested</u>	<u>Percent Positive</u>	<u>Range of Titer</u>	<u>Geometric Mean Titer</u>
Adult AIDS Patients	21/35	60	10-520	44
Pediatric AIDS Patients	9/9	33	80-180	117
Adult ARC Patients	28/35	80	17-560	88
Healthy Homosexuals	2/12	17	130-340	210
Healthy Heterosexuals	0/20	0	-	-
Heterosexual Partners of AIDS Patients ^b	1/3	33	78	-
Mothers of Pediatric AIDS Patients ^c	0/2	0	-	-
Siblings of AIDS Patients ^d	1/2	50	55	-
Patients with Acute Mononucleosis	1/4	25	13	-
Patient with Sarcoidosis	0/1	0	-	-

AIDS = acquired immunodeficiency syndrome; ARC = AIDS related complex

^a All sera were screened for virus neutralizing antibody at a 1:10 dilution. Those sera possessing activity were further titered as described in Example 2. Antibody titer is defined in the footnote to Table 1.

^b All 3 individuals were positive for HTLV-III antibodies by the ELISA and Western blot assays.

^c Sera from these 2 foster mothers were negative for HTLV-III antibodies by the ELISA and Western blot assays.

^d The positive sibling was also antibody positive by the ELISA and Western blot assays.

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EXAMPLE 1

For virus absorption experiments, 62 ml of cell-free virus supernatant containing 2 to 5×10^8 virus particles/ml were pelleted as described in Example 2. The viral pellet was resuspended in 100 μ l of a 1:10 dilution of serum to be absorbed and incubated overnight at 4°C. The virus was again pelleted by centrifugation and the absorbed serum was saved for titration of virus neutralizing antibody activity. Sera were similarly absorbed on pellets of washed 10^7 cells and titered.

Values for percent of HTLV-III p24-positive cells were normalized to the level of infection obtained in the presence of a standard negative serum. Antibody titers were then expressed as the reciprocal of the serum dilution at which virus infection was 60% of that obtained in the presence of a standard negative serum.

EXAMPLE 2

In the method for screening human sera for HTLV-III neutralizing antibodies, media containing 2 to 5×10^8 HTLV-III particles/ml were harvested from H9/HTLV-III cells. The amount of virus initially used was determined by titrating a virus preparation and selecting an amount for the assay which would achieve 50 to 80% of infected H9 cells by 3 days post infection. This in general required a substantial excess of virus particles per target cell. Cells were removed by low-speed centrifugation and the virus-containing supernatant was centrifuged for 3 hours at 32,000 x g. The viral pellets were resuspended in a total volume of 2.25 ml media (RPMI 1640 containing 20% fetal calf serum and penicillin/streptomycin). Uninfected H9 cells were washed in media and incubated for 20 minutes at room temperature in media containing 2 μ g/ml polybrene. The cells were washed in media and resuspended at a concentration of 4×10^6 /ml in media. Sera to be tested were heat inactivated at 56°C for 30 min. and filter sterilized. For each assay 20 μ l of virus suspension and 20 μ l of a 1:10 dilution of serum

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was mixed and incubated in a well of a microtiter plate for 1 hr at 4°C and then 15 min. at room temperature. H9 cells (10 µl) were added to each well and incubation was continued for 1 hr at 37°C. Aliquots (15 µl) of each mixture were plated into 200 µl media in duplicate wells of another microtiter plate. Cultures were incubated at 37°C in a 5% CO₂ incubator. After 3 days, cultures in individual wells were removed, washed 2 times with phosphate buffered saline (PBS) and once with PBS:water, 1:1. Cells were suspended in approximately 30 µl of the same solution and 5 to 10 µl aliquots were spotted on 8-well toxoplasmosis slides for an indirect fixed-cell immune fluorescent assay using a monoclonal antibody to HTLV-III p24.

Sera exhibiting neutralizing antibody activity and a 1:10 dilution were subsequently serially diluted and the assay was repeated to determine antibody titer.

In the following claims and in the specification sera refers to sera containing a substantial quantity of anti-HTLV-III. This includes sera from adult and pediatric AIDS and ARC patients and healthy homosexual (see Table 2). The geometric titer ranges from about 44 to 210.

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CLAIMS

1. A method of neutralizing HTLV-III virus infectivity, comprising:

- 5 (a) treating said virus with natural human antibodies in sera containing antibody to HTLV-III; and
(b) assaying residual viral infectivity to determine the measure of protection afforded by the natural antibodies.

10 2. The method of Claim 1, wherein the assaying is carried out with a monoclonal antibody specific for HTLV-III.

3. The method of Claim 2, wherein the monoclonal antibody is anti-HTLV-III p24.

15 4. A method of neutralizing HTLV-III virus infectivity, comprising:

- (a) treating said virus with sera containing natural human antibodies;
(b) infecting and incubating the resulting culture with H9 cells; and
20 (c) assaying for residual infectivity with a monoclonal antibody specific for HTLV-III or HTLV-III p24.

5. The method of Claim 4, wherein the monoclonal antibody is specific for HTLV-III p24.

25 6. The method of Claim 4, wherein the monoclonal antibody is specific for HTLV-III.

7. The method of Claim 4, further comprising selecting an amount of virus for assay such that 50 to 80% of the H9 cells are infected.

30 8. A method of assaying the protective effects on HTLV-III virus by addition of sera containing neutralizing antibodies, comprising measuring residual viral infectivity by addition of a monoclonal antibody specific for HTLV or p24 HTLV.

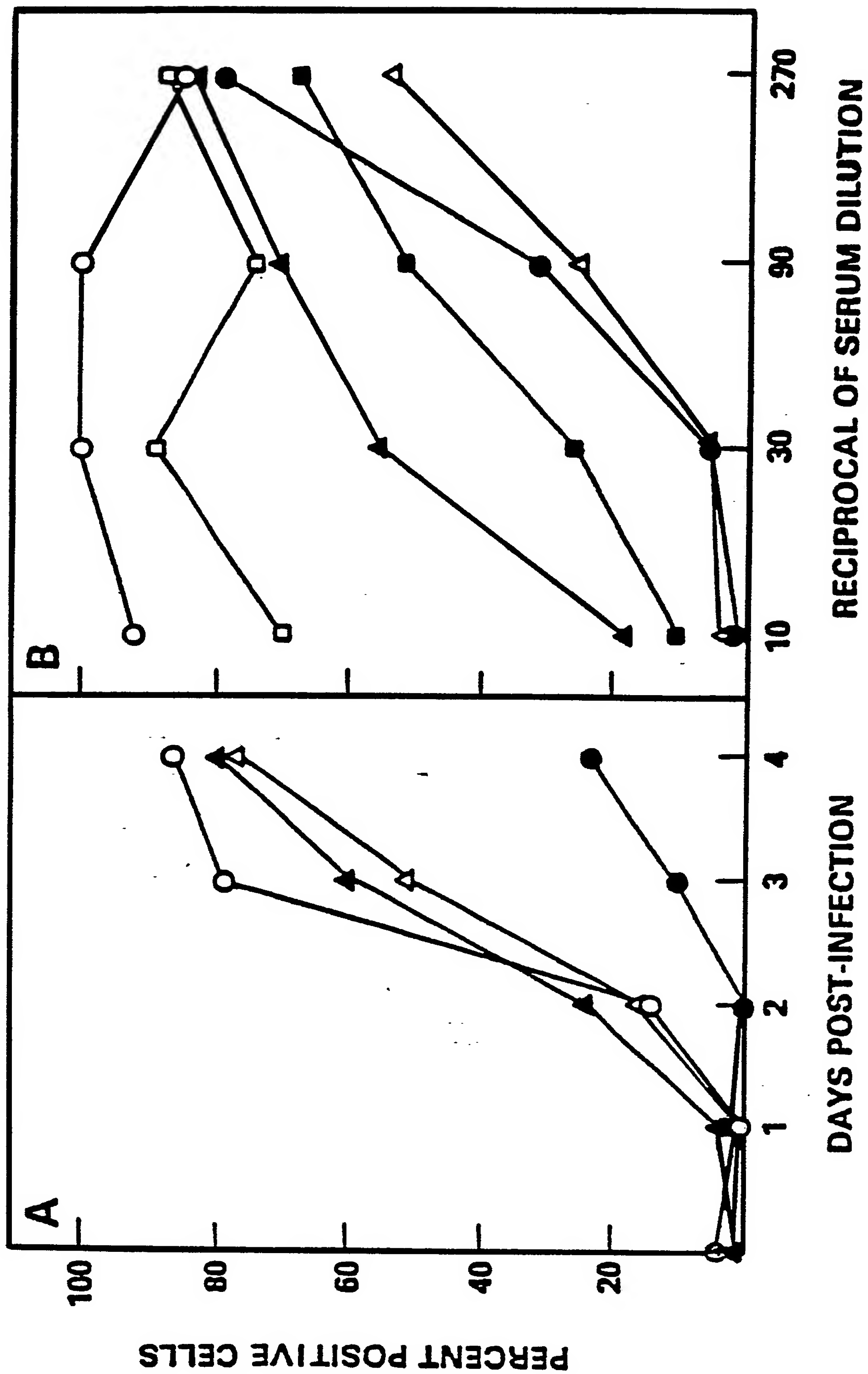
35 9. The method according to Claim 8, further

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comprising a hold period of three days prior to the addition of the monoclonal antibodies to allow for viral infection.

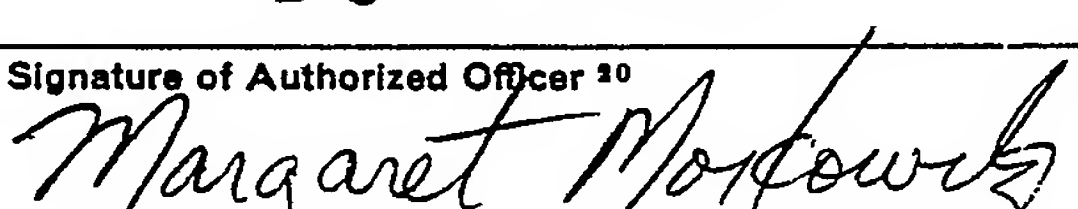
5 10. The method according to Claim 8, wherein an immune fluorescent assay is used for reaction of an antigen and the monoclonal antibody.

1 / 1



INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00217

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC INT. C1. ⁴ C12Q 1/70 U.S. 435/5		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/5, 7, 29, 68, 172.2, 240 436/506, 510, 811	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
COMPUTER DATABASE: BIOSIS; FILE 5 (1981-1985)		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y, P	US, A, 4,520,113, Published 28 May 1985, Gallo et al.	1-10
X	N, Journal of Immunological Methods, Volume 76, Issued 1985, Pages 171-183, McDougal et al, "Immunoassay for the Detection and Quantitation of Infectious Human Retrovirus, Lymphadenopathy-Associated Virus (LAV)."	1-10
Y	N, Magyar Onkologia, Volume 29 Issued 1985, Pages 11-18, Nagy et al, "Detection of Receptors and Neutralizing Antibodies of the Human T-Cell Leukemia Virus By Pseudo- type Method."	1-10
Y	N, Science, Volume 224, Issued May 1984, Pages 506-508. Sarngadharan et al, "Antibodies Reactive with Human T-Lympho- tropic Retroviruses (HTLV-III) in the Serum of Patients with Aids."	1-10
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ³	
27 February 1986	10 MAR 1986	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US		

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	N, <u>Microbiology</u> , Third Edition, Harper and Row, Publishers, Philadelphia, Published 1980, Davis, B. D. et al, Eds., Pages 1018-1030, "Viral Immunology."	1-10

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X,Y	N, Proceedings of the National Academy of Sciences, USA, Volume 81, Issued May 1984, Pages 2886-2889, Clapham et al, "Pseudotypes of Human T Cell Leukemia Virus Type 1 and Type 2 Neutralization by Patients Sera."	1-7,8-10
Y	N, Journal of Experimental Medicine, Volume 154, Issued December 1981, Pages 1957-1964, Robert-Guroff et al, "Detection of The Human T Cell Lymphoma Virus p19 in cells of some patients with cutaneous T Cell Lymphoma and Leukemia Using a Monoclonal Antibody."	10

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.